

5. YERSINIOSIS: TAXONOMY, PHYLOGEOGRAPHY, POLYMORPHISM OF PATHOGENICITY FACTORS AND SELECTIVE VIRULENCE

5.1 *YERSINIA PESTIS* VOLE'S STRAINS: TAXONOMY, PHYLOGEOGRAPHY, POLYMORPHISMS OF PATHOGENICITY FACTORS AND SELECTIVE VIRULENCE

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Strains of *Yersinia pestis*, the causative agent of plague, can be divided into those possessing universal virulence and isolates conditionally pathogenic for guinea pigs and humans.

Purpose and objectives of our study were intraspecific classification, analysis of phylogeography, determination of polymorphism of pathogenicity factors and identification of selective virulence factors using postgenomic technologies.

More than 100 isolates from natural plague foci of the former USSR and Mongolia were studied with (i) DFR-, IS100-, CRISPR-, MLVA25-, and SNP-typing, (ii) sequencing of genes coding for several pathogenicity factors, (iii) 50 of the strains were underwent the whole genome sequencing, (iv) the whole genome sequences, mRNA or protein spectra synthesized at 37°C *in vitro* or in a dialysis chambers placed into guinea pig peritoneum cavities were compared in the bv. ulegeica strains subcultures differing in subcutaneous virulence for guinea pigs by more than five orders of magnitude, (v) knockout mutagenesis and subsequent complementation were used to assess the significance of identified potential selective virulence factors.

Y. pestis division into two subspecies differing in epidemiological significance is justified. DFR-, IS100-, CRISPR-, MLVA25-, and SNP-typing had very similar clustering ability. Polymorphism of the classic pathogenicity factors was not associated with selective virulence. One or several genotypes were isolated only in certain natural foci. Strain subcultures dramatically differing in their subcutaneous virulence for guinea pigs had identical nucleotide sequences in their genomes but fluctuated in mRNA and protein spectra. Most of the observed differences in the spectra of synthesized mRNA and proteins did not affect virulence for both mice and guinea pigs. According to our research, the main candidates for the role of selective virulence factors are methionine ABC transporter lipoprotein (WP_038931127.1) and class II fructose-bisphosphate aldolase (WP_002209962.1).

A rational variant of bringing the taxonomy of the plague microbe in accordance with the rules of the International Code of Bacterial Nomenclature and evolutionary taxonomy is proposed. Two new potential molecular targets for the prevention and treatment of plague are found.

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5.2 CREATING A SPECTRA LIBRARY FOR SPECIFIC MALDI-TOF MASS SPECTROMETRY IDENTIFICATION OF *YERSINIA ENTEROCOLITICA*-LIKE SPECIES

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Phenotypic identification of *Y. enterocolitica*-like species by biochemical tests is laborious and not always reliable. Among these species, identification of *Y. bercovieri*, *Y. mollaretii*, *Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia* is a problem for clinical microbiologists, and usually misidentify them as *Y. enterocolitica*, an important food-borne pathogen. In last study, 1138 *Yersinia* strains isolated in Russia were analyzed by MALDI ToF mass spectrometry (MALDI ToF MS) using Microflex™ LT (Bruker Daltonics, Germany). We demonstrated the accurate MALDI ToF MS genus identification, and *Y. pseudotuberculosis* and *Y. enterocolitica* species identification (100% of strains).

However only 22.1% of strains 217 *Y. enterocolitica*-like species were further reliably identified to the species level (Score Value ≥ 2.3 correspond to highly probable species identification). Other *Y. enterocolitica*-like strains were misidentified as *Y. enterocolitica*.

The aim of this study was to create in-house spectra library for correct identifying *Y. enterocolitica*-like species.

MALDI ToF MS identification of the *Y. enterocolitica*-like species is limited by small number of their reference spectra available in the MALDI Biotyper database. The mass-spectra from 60 well-characterized *Y. enterocolitica*-like strains (53 were isolated in Russia, 7 are Institute Pasteur reference strains) were used to generate a project library: *Y. kristensenii* (n = 20), *Y. intermedia* (n = 15), *Y. frederiksenii* (n = 14), *Y. aleksiciae* (n = 5), *Y. bercovieri* (n = 4), *Y. mollaretii* (n = 2). To select strains, the obtained spectra of 263 *Y. enterocolitica*-like strains were used for cluster analysis. It was shown that *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii* spectra forms a few subclusters. So for library creation we selected the strains of spectra different subclusters.

For applying in-house spectra library additional verified strains *Y. intermedia* (n = 20), *Y. frederiksenii* (n = 15), *Y. kristensenii* (n = 20) and *Y. enterocolitica* (n = 20) were used. All strains were accurate identified to the species level with Score Values ≥ 2.3.

Thus the creating in-house spectra library allows to increase the specificity of *Y. enterocolitica*-like species MALDI ToF MS identification.

5.3 EPIDEMIOLOGICAL AND CLINICAL PECULIARITIES OF PSEUDOTUBERCULOSIS OUTBREAKS

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Retrospective analysis of 29 pseudotuberculosis outbreaks in Siberia and at the Far East demonstrated the following patterns:

- outbreaks mainly belonged to short-term type I lasting no more than one incubation period;

- they were mostly registered in the children's organized groups (86,2%) including 12,0% cases in child-care facilities;
- they occurred more frequent in autumn-winter (46,2%) and spring (38,4%) periods of a year and were associated with consumption of vegetable salads in 72,4% cases;
- violation of sanitary-and-hygienic mode and technology of meal preparation from uncooked vegetables was noted in all outbreaks;
- laboratory confirmation of the diagnosis was set in 39,5% of the patients by PCR assay in the first days; bacteriological and serological diagnosis was confirmed in 14,1 and 64,6% after 2–3 weeks;
- *Yersinia pseudotuberculosis* was revealed in the population of synanthropic and wild small mammals in 52,9%, in transmission factor — in 46,7% from the total number of the studied outbreaks;
- all epidemic *Y. pseudotuberculosis* strains were O:1b serotype, possessed *ypm* gene of super-antigen, lacked of a high pathogenicity island (HPI), and belonged by plasmid content to single plasmid (pYV 47 MDa) and two-plasmid (pYV 47 MDa and pVM 82 MDa) strains with the identical frequencies;
- the peculiarity of clinical manifestation of pseudotuberculosis caused by *Y. pseudotuberculosis* with two-plasmid and chromosomal *tcpYI* gene (phagocytosis inhibitor) was the presence of the intoxication symptoms, fever, rash, damage of gastrointestinal tract, liver and joints with prevalence of medium-severe and severe course specific for Far Eastern scarlet-like fever (FESF);
- we discovered one more form of FESF clinical course caused by *Y. pseudotuberculosis* with pYV plasmid and lacking *tcpYI* gene. In this case all observed symptoms were poorly expressed, and pseudotuberculosis was developed in "minor" easier form mainly in children.

The revealed peculiarities of pseudotuberculosis outbreaks are necessary to take into consideration in epidemiological surveillance.

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INTRASPECIFIC DIVERSITY OF *YERSINIA PESTIS* CHAPERONE/USHER SECRETION APPARATUSES

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The Post-Antibiotic Era requires replacement of antibiotics with alternative antibacterials aimed at alternative molecular targets. One of such alternative approaches to treat infections are remedies targeting virulence. *Yersinia pestis* as many other Gram-negative bacterial pathogens use the chaperone/usher (CU) pathway to assemble virulence-associated surface fibers termed pili or fimbriae. *Y. pestis* has two well-characterized CU operons: the *caf* genes coding for the F1 capsule and the *psa* genes coding for the pH 6 antigen. There are eight additional CU secretion systems capable of assembling *Y. pestis* pilus fibers. When choosing new targets for effective treatment of infectious diseases, it is necessary to search for pathogenicity factors possessing structural conservatism, since polymorphism gives pathogens the opportunity to evade interaction with the drug.

Searches and comparisons of amino acid sequences of CU proteins from *Y. pestis* strains belonging to SNP-types 0.PE2, 0.PE3, 0.PE7, 0.PE4, 0.PE5, 1.ORI, 1.ANT, 2.ANT, and 2.MED were conducted using the databases of the National Center for Biotechnology Information

(<http://www.ncbi.nlm.nih.gov>) by MAUVE (<http://darlinglab.org>), BLAST (<https://blast.ncbi.nlm.nih.gov>), ProtParam tool (<https://web.expasy.org/protparam>), and protein sequence analysis (http://molbiol.ru/scripts/01_18.html). The usher genes for two of chaperone/usher pathways (*y1539-1544* and *y4060-4063*) were disrupted in all of the studied *Y. pestis* strains by an insertion sequence or premature stop codon, and thus these pathways are not expected to be functional. The phylogenetic-group-specific polymorphisms of amino acid sequences of the proteins from the *Y. pestis* CU secretion systems is inherent in five ushers (*y0562*, *y1858*, *y1871*, *y2390*, *y3480*), three molecular chaperone (*y2392*, *y3479*, *caf1M*) and three adhesin subunits (*caf1*, *y2388*, *y3478*). These polymorphic proteins are excluded from the list of potential *Y. pestis* molecular targets.

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THE OUTER MEMBRANE PROTEIN A (*ompA*) OF *YERSINIA PESTIS* IS NOT REQUIRED FOR VIRULENCE IN MICE AND RATS

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The plague bacterium *Yersinia pestis* has a number of well-described strategies to protect itself from the both cellular and humoral factors of the host's innate immunity. OmpA in several pathogens has been shown to mediate resistance to complement and antibacterial peptides, as well as play a role in invasion and intracellular survival. In this study, we sought to determine whether deletion of the *ompA* would render fully virulent *Y. pestis* strain attenuated in the mouse and rat models of plague.

Y. pestis ΔompA mutant was constructed using the knockout mutagenesis. SDS-PAGE and Western blot analyses with anti-OmpA serum showed the absence of OmpA in *Y. pestis ΔompA* cell lysates and outer membranes preparations. We could not detect any differences between *Y. pestis* wild type strains and their *ΔompA* derivatives using a serum killing assay. The OmpA deficient mutants were 2 times less resistant to bactericidal action of polymyxin B as compared with the wild type strains. To assess the biological significance of OmpA in fully virulent *Y. pestis* strain *in vivo*, studies in a mouse and rat models of bubonic and pneumonic plague were performed. Inbred mice and rats were infected subcutaneously or intranasally to mimic bubonic or pneumonic plague and observed for 21 days. Comparative study of the virulence of *Y. pestis* mutant strains using subcutaneously and intranasally challenged mice and intranasally challenged rats did not reveal differences in their LD₅₀. The average survival time of mice and rats that succumbed to infection with the strain 231 or its isogenic derivative did not differ from each other. The estimated LD₅₀ of the *ompA* mutant for subcutaneously challenged rats was approximately 10-fold higher than the LD₅₀ of the wild type 231 strain.

The main outcome of our investigation is the finding that the loss of the ability to produce OmpA antigen did not influence virulence of *ΔompA* mutant of *Y. pestis*. This argues against the usefulness of using OmpA as a molecular target for plague prophylaxis and therapy.

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